Characterization of the Major Metabolite of Sampangine in Rats

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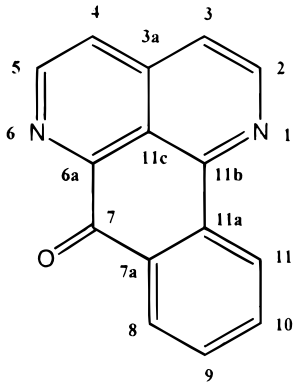
Sampangine (**1**) is a plant-derived antifungal copyrine alkaloid extracted from the stem bark of *Cananga odorata*. Although it possesses potent in vitro antifungal activity, **1** is devoid of significant and reproducible in vivo activity in a mouse model of cryptococcosis. Speculating that the lack of in vivo activity could be due to metabolism, a study was undertaken to begin to develop an understanding of the pharmacokinetics, and particularly metabolism of **1**. Following intraperitoneal administration of **1** to rats, urine was collected, extracted, and chromatographed over a reversed-phase C_{18} silica column to yield the major metabolite, SAM MM1 (**2**), which was identified by NMR and MS to be an *O*-glucuronide conjugate of sampangine. In addition, two other unstable, structurally uncharacterized minor metabolites were produced, as evidenced by HPLC analysis. Evaluation of the antifungal and antibacterial activities of **2** showed it to have remarkable in vitro activity against *Cryptococcus neoformans*.

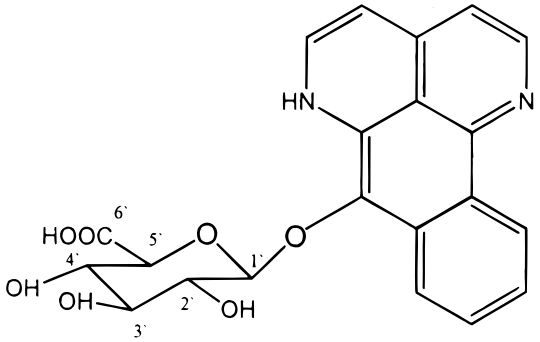
The discovery and development of novel antiinfectives, particularly antimycotic agents, is an important priority since mortality and morbidity associated with AIDS is often due to opportunistic infections (OI), including fungal infections. The urgency for such research is underscored by many problems encountered in the treatment of such infections in AIDS patients, including long duration of therapies, more frequent and severe drug toxicities, the relative ineffectiveness of the available systemic antifungal agents, and the development of resistance to the existing agents. These significant shortcomings of existing therapies have intensified the search for new, more effective, and less toxic prototype antifungal agents.[1]

As a part of an extensive program aimed at the discovery and development of prototype antibiotics from higher plants, we reported previously the isolation, structure elucidation, and in vitro antifungal activity of the novel copyrine alkaloid 3-methoxysampangine from the root bark of the west African tree *Cleistopholis patens* (Benth.) Engl. & Diels (Annonaceae).[2] Later, in 1992, the synthesis and in vitro antimycotic/antimycobacterial activities of several analogues and derivatives were reported.[3] Due to the scarcity of 3-methoxysampangine (0.000156% w/w yield from the natural source), the parent compound of the class, sampangine (**1**), was selected for study as a model for understanding this class of alkaloids. Sampangine, originally reported as a natural product from the stem bark of *Cananga odorata* Hook. f. and Thomas (Annonaceae),[4] has also been synthesized in 26% yield by Bracher.[5] In our laboratories, sampangine was shown to be as active in vitro as 3-methoxysampangine against *Candida albicans* and *Cryptococcus neoformans*.[3] However, sampangine does not show significant, reproducible in vivo activity.[6] In light of its exceptional and potent in vitro activity, it was speculated that the lack of in vivo activity could be due to metabolism. We report now the results of rat metabolism studies of sampangine.



Sampangine (**1**)

In the present study, we have obtained one major metabolite designated SAM MM1 (sampangine mammalian metabolite) (**2**) in the urine of male Wistar rats.



SAM MM1 (**2**)
(Sampangine β-glucuronic acid)

SAM MM1 was identified on the basis of a comparison with microbial metabolites (**3**, **4**) that are similar, but not identical.[7] Mechanisms for metabolite formation based on metabolite structure and stability are proposed. Additionally, two very polar minor metabolites were also observed by HPLC, but these were unstable and could not be isolated in quantities sufficient for structure determination. These minor metabolites accounted for approximately 13% of the administered dose.

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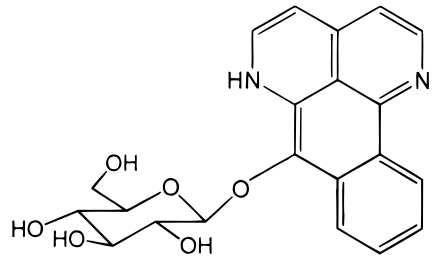
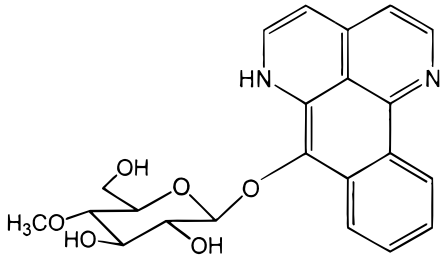


Sampangine β-glucopyranoside (**3**)



Sampangine-4-*O*-methyl- β-glucopyranoside (**4**)

SAM MM1 (**2**) was obtained by reversed-phase chromatography of the *n*-BuOH extract of the urine of rats dosed with sampangine (**1**). This metabolite represented approximately 33% of the administered dose in the 24-h urine collection. The physiochemical properties of SAM MM1 were similar, but not identical, to those of sampangine metabolites produced by microbial systems.[7] However, SAM MM1 is red in color (in acidic medium) and more polar than the microbial metabolites.

The thermospray mass spectrum of SAM MM1 showed no molecular ion peak, indicating that the metabolite decomposed thermally during analysis. The high-resolution electrospray impact (HRESI) mass spectrum of SAM MM1 revealed a molecular ion peak at *m*/*z* 411.1188 ($M + H^+$), consistent with the molecular formula $C_{21}H_{18}N_2O_7$. This molecular weight was the sum of sampangine [232 amu] and a moiety of 178 mass units (consistent with glucuronic acid). HRESI analysis, and the fact that glucuronidation is one of the three common mammalian phase II metabolic reactions, provided strong evidence that SAM MM1 (**2**) is an *O*-glucuronide of sampangine. Confirmation of the structure of **2** was achieved through NMR analyses.

The anomeric proton, which resonates at δ 4.85 ppm (Table 1), shows a large coupling constant (J = 8.0 Hz), indicating that the glucuronic acid binds to the aglycon in a β-glycosidic linkage, similar to that observed for the microbial metabolites and to other published data.[7,8] The nuclear Overhauser effect between the anomeric proton and the proton resonating at δ 8.68, assigned to H-8 (J = 7.3 Hz), led to the conclusion that SAM MM1 is an *O*-glycoside in which the aglycon part is coupled with sampangine through its reduced carbonyl group (C-7). Since the anomeric proton was distinguishable, other sugar protons were assigned unambiguously from the COSY spectrum (phase-sensitive DQ-filtered COSY). Likewise, other aromatic protons were assigned from the COSY spectrum starting from the preassigned H-8 (Table 1). Other ROESY cross-peaks were also seen for proton pairs H-2 and H-3, H-3 and H-4, H-4 and H-5, H-8 and H-9, H-9 and H-10, and H-10 and H-11. The identity of glucuronic acid as the glycon portion was confirmed from the coupling constants, $J_{2',3'}$, $J_{3',2}$, $J_{3',4'}$, $J_{4',3'}$, $J_{4',5'}$, and $J_{5',4'}$, all of which were larger than 7.0 Hz (Table 1), indicating the 1,2-*trans*-diaxial relationship between each consecutive pair of protons. Furthermore, the existence of NOEs between H-1′ and H-3′, H-1′ and H-5′, H-3′ and H-5′, and H-2′ and H-4′ suggests 1,3-*cis*-diaxial interactions between these protons.

Table 1. ^{1}H and ^{13}C NMR Assignments of SAM MM1 (**2**)

carbon number	δ_H	δ_C
2	8.20 (1H, d, J = 6.6)	145.3 (1)
3	6.80 (1H, d, J = 6.6)	110.1 (1)
3a		143.0 (0)
4	6.05 (1H, d, J = 7.3)	100.8 (1)
5	7.38 (1H, d, J = 7.3)	140.0 (1)
6a		131.5 (0)
7		135.0 (0)
7a		131.5 (0)
8	8.68 (1H, d, J = 7.3)	122.2 (1)
9	7.75 (1H, dd, J = 7.3, 7.3)	129.5 (1)
10	7.55 (1H, dd, J = 7.3, 7.3)	124.3 (1)
11	8.62 (1H, d, J = 7.3)	122.5 (1)
11a		128.0 (0)
11b		143.0 (0)
11c		123.0 (0)
1′	4.85 (1H, d, J = 8.0)	106.2 (1)
2′	3.80 (1H, dd, J = 8.0, 8.0)	74.0 (1)
3′	3.55 (1H, dd, J = 8.0, 8.0)	76.8 (1)
4′	3.65 (1H, dd, J = 8.0, 8.0)	75.08 (1)[a]
5′	3.65 (1H, d, J = 8.0)	72.0 (1)[a]
6′		176.8 (0)

[a] Interchangeable

Table 2. In Vitro Antifungal Activities of Sampangine (**1**) and SAM MM1 (**2**)

	bioautography[a]		agar well diffusion[a]		MIC[b]	
compound	Ca[c]	Cn[d]	Ca[c]	Cn[d]	Ca[c]	Cn[d]
sampangine (**1**)	10	35	10	35	3.12	0.20
SAM MM1 (**2**)[e]	NT	35	6	26	>25	>25

[a] Activity expressed as zone of inhibition measured in mm (test concentration 100 μg/mL). [b] Activity expressed as minimum inhibitory concentration (μg/mL) using 2-fold serial broth macrodilution technique. [c] Ca: *C. albicans* NIH B311 in yeast nitrogen broth. [d] Cn: *C. neoformans* ATCC 52657 in Mycophil broth. [e] Tested in Eugon using a microplate method.

The HMQC spectrum of SAM MM1 (**2**) showed 21 carbon resonances. These resonances were comparable to those of the microbial metabolites, except for one signal resonating at δ 176.8 ppm, which is consistent with the presence of the carbonyl of a carboxylic acid (glucuronic acid moiety) (Table 1). Comparison of the ^{1}H NMR and ^{13}C NMR resonances of the rat metabolite and those of the microbial metabolite (**5**) confirm **2** for SAM MM1.

Although evaluation of in vitro antifungal activity of the mammalian metabolites in agar well diffusion assays showed inhibition zones comparable to sampangine (**1**), the MIC values of SAM MM1 (**2**) in a broth microdilution assay are much higher (less active) than sampangine against *C. neoformans* and *C. albicans* (Table 2). This can possibly be explained by the high water solubility of the glucuronide, limiting its availability to the yeast in the broth. Although sampangine is marginally to moderately active against the bacterium *Staphylococcus aureus*, SAM MM1 is inactive as an antibacterial.

In conclusion, a major mammalian metabolite of sampangine (**1**), SAM MM1, has been isolated and characterized as the *O*-glucuronide conjugate (**2**). Evaluation of the antifungal and antibacterial activities of SAM MM1 shows it to have remarkable activity against *C. neoformans*, but little or no activity against other pathogens. Thus, it appears that metabolism per se may not be wholly responsible for the lack of in vivo efficacy of sampangine. Further

studies on other pharmacokinetic parameters (bioavailability, distribution, clearance, etc.) are in progress.

Experimental Section

General Experimental Procedures. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker DMX-600 spectrometer equipped with a microprobe and operating at 600 MHz for δ_H and 150 MHz for δ_C. Both ^{1}H and ^{13}C NMR spectra were recorded in deuterated methanol, and the chemical shift values are expressed in parts per million (ppm) relative to the internal standard, tetramethylsilane. For the ^{13}C NMR spectra, the numbers 0, 1, 2, 3 refer to the number of attached protons as determined by the attached proton test (APT) and the distortionless enhancement through polarization transfer experiment (DEPTGL). Two-dimensional NMR data were obtained using the standard pulse sequences of the Bruker DMX-600 for phase-sensitive DQ-filtered COSY, heteronuclear multiple quantum coherence spectroscopy (HMQC), heteronuclear multiple bond correlation spectroscopy (HMBC), nuclear Overhauser and exchange spectroscopy (NOESY), and rotating-frame Overhauser enhancement spectroscopy (ROESY). High-resolution electrospray impact (HRESI) mass spectral analyses were carried out at Bruker Analytical Systems, Inc., Mass Spectrometry Division, Bellerica, MA. HPLC purification was done using Waters 600 automated gradient controller equipped with Waters 486 tunable absorbance detector and Waters 510 HPLC pumps connected to a silica gel column YMC-pack SIL, 250 × 10 mm i.d., 5−5 μm, 120 Å, and eluting with MeOH−$CHCl_3$−NH_4OH (19.5:80:0.5). TLC analyses were performed on precoated silica gel G-25 UV_{254} plates (0.25 mm, Machery-Nagel Düren) using MeOH−$CHCl_3$−NH_4OH (2.95:7.0:0.05) as the developing solvent.

Chemicals. Sampangine (**1**) used in this study was obtained through total synthesis in our laboratories using the previously reported methodology of Bracher.[5] All other chemicals were reagent grade or higher.

Animal Studies.[9] A male Wistar rat weighing 250 g was acclimated by maintenance in a metabolism cage with access to food and water. One day later, the animal was injected intraperitoneally with sampangine (**1**) (a 10% suspension in DMSO) at a dose of 80 mg/kg body weight (total sampangine dose 20 mg). Urine was collected over 24 h. The urine assumed a red coloration within 1 h after the dosing, reaching a peak intensity of red color after 6 h, and continuing for about 48 h post-dosing. The collected urine (20 mL) was extracted with H_2O-saturated *n*-BuOH (4 × 20 mL). The combined extracts were evaporated in vacuo at 40 ° C to afford a red residue (891 mg).

Isolation of Mammalian Metabolites. The red residue (891 mg) was chromatographed over reversed-phase Si gel [C_{18}, 40−63 μm, EM Science; 40 g column (2 × 22.5 cm)], with MeOH−H_2O (10:0 to 8.5:1.5) mixture as eluent; 20 mL fractions were collected to afford two pooled fractions from which SAM MM1 (**2**) was isolated and two minor metabolites were detected.

TLC of fractions 1−9 showed a single red spot with R_f 0.13, and these fractions were combined and evaporated to dryness to give 6.6 mg of SAM MM1 (**2**) as a red amorphous powder (18.7% yield). The ^{1}H and ^{13}C NMR resonances are presented in Tables 1 and 2, respectively. HRESIMS: *m*/*z* 411.1188 (calcd for $C_{21}H_{18}N_2O_7 + H^+$, 411.1192).

TLC of fractions 11−23 showed a single spot with R_f 0.46, and these fractions were combined to give 1.4 mg of a red amorphous powder, which when purified by HPLC, showed two closely related metabolites. No spectral data could be obtained on these products due to their low quantities and instabilities.

Biological Methods. In vitro activity evaluation versus *C. albicans* NIH B311 and *C. neoformans* ATCC 52657 was accomplished by using previously described bioautography[10] and agar well-diffusion assay[11] methods. Minimum inhibitory concentrations (MIC) were determined using a 2-fold serial broth macrodilution assay.[12] Following incubation (37 °C, 48 h for *C. neoformans*; 24 h for *C. albicans*), the MIC was taken as the lowest concentration inhibiting growth of organism based on visual assessment. Sampangine (**1**) was used as a positive control for both qualitative and quantitative assays.

In vitro quantitative antibacterial activity was evaluated using a colorimetric oxidation−reduction assay that involves the addition of Alamar blue dye as an indicator of metabolic activity of the organism.[13] The activity is expressed as MIC (μg/mL). Sampangine and SAM MM1 were also tested for their activities against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilus* ATCC 6333, and *Escherichia coli* ATCC 10536. Streptomycin was used as a positive control for antibacterial assays.

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